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14. ABSTRACT We have made significant progress at the University of Colorado site with the other teams at Oregon Health and Science University and City of Hope. The major objective of this project is to develop novel strategies to enhance the protective effects of anti-tumor T cells in breast cancer (bc) patients based on the hypothesis that partially protective anti-tumor T cells exist within in most bc patients. We previously determined that the sequence of the TCR of T cell clones expanded in culture must be compared with those isolated ex vivo because their TCR repertoires are skewed in culture. Since the T cells cannot be cultured, we developed an emulsion PCR assay to sequence the alpha and beta chain of single T cells in one reaction. Using this assay we identified TCRs enriched in tumor infiltrating lymphocytes, and found at low levels in the peripheral blood. A number of TCRs were found in multiple patients suggesting that targeting these TCRs may be most efficient and that they are restricted by the HLA-A2 molecule, the HLA that the bc patients all have in common. With this technique in hand, we are positioned to screen known bc antigens and peptide/MHC libraries for new bc epitopes and mimotopes.					
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1. INTRODUCTION:

We have made significant three years into the Multi-Team Project ENHANCING THE BREADTH AND EFFICACY OF THERAPEUTIC VACCINES FOR BREAST CANCER (months 25 to 36). The major objective of this project is to develop novel strategies to enhance the protective effects of anti-tumor T cells in breast cancer patients. This is based on the hypothesis that partially protective anti-tumor T cells exist within most breast cancer (bc) patients. We previously determined that the TCR sequence of T cell clones expanded in culture must be compared and matched with those isolated ex vivo for the identification of epitopes and mimotopes; i.e., the TCR repertoire becomes significantly skewed in culture. Thus, we developed an emulsion PCR assay to sequence the alpha and beta chain of single T cells in one reaction. This method is our most significant reportable out come this year. Using this new method we identified TCRs enriched in tumor infiltrating lymphocytes, and found at lower frequencies in the peripheral blood. A number of TCRs were found in multiple patients suggesting that these epitopes are HLA-A2-restricted and targeting these TCRs may lead to a more generalizable vaccine than those only good for one patient. Two patients with similar TCR profiles had the same subtype of cancer, luminal A, although other patients with this subtype had different repertoires. With this technique in hand, we are positioned to screen known bc antigens and peptide/MHC libraries for new bc epitopes and mimotopes.

The teams meet regularly on monthly conference call (organized by the OHSU group) and in person bi-annually. Since our last report, we met on 2-24-2014 at the City of Hope and on 8-26-2014 in Portland.

2. KEYWORDS

Breast cancer, tumor antigens, T cell receptor, cancer vaccine

3. OVERALL PROJECT SUMMARY

Task 1. Generate reagents and identify conditions for experiments to follow: months 1- 40, Lee, Slansky, and Spellman This project summary is arranged in the order of the Statement of Work (revised 1-1-12). As planned, we are still performing task 1, details are included in the other tasks. The tasks that we have not included are either being performed by the other team, or have not been started yet.

Task 2. Enroll 100 patients with all major breast cancer subtypes from City of Hope and University of Colorado.

We have acquired 19 samples from Dr. Virginia Borges at the UC Anschutz Medical Campus (AMC), 7 were used in the sequencing experiments; and 21 samples Dr. Peter Lee at the City of Hope, all were used in the sequencing experiments. A summary of the patients is provided in [Table 1](#).

Patient*	Subtype	Stage	HLA type	CD8 yield	CD4 yield	Sample use of CD8 TILs**
UC0147	Basal-like	Stage I T1N0M0	<u>HLA-A*02:01</u> HLA-DR*13:03	117,750	92,250	Used to analyze function of TIL and troubleshoot sequencing
UC0152	Luminal A	Stage I T1N0M0	Not done	Not done	Not done	Tissue too small, not used
UC0157	Basal-like	Stage II T2N0M0	<u>HLA-A*02:01</u> HLA-DR*11:01	<6,000	<6,000	Used to troubleshoot sequencing
UC0197	Luminal B	Stage I T1N0M0	<u>HLA-A*02:01</u> HLA-DR*13:01	<4,000	<4,000	Sequencing
UC0198	Luminal B	Stage II T2N1M0	HLA-A*23 HLA-DR*07:01	68,000	24,000	TIL RNA stored for future use
UC0200	Luminal A	Stage III T3N3M0	<u>HLA-A*02:01</u> HLA-DR*01:01	T:30,000 LN: 37,500	T:15,000 LN: 62,500	Sequencing, TDLN included
UC0202	Luminal A	Stage II T2N1M0	HLA-A*01:01 HLA-DR*07:01	<4,000	<4,000	TIL RNA stored for future use
UC0205	Luminal A	Stage II T2N1M0	HLA-A*11:02 HLA-DR*09:01	<6,000	<6,000	TIL RNA stored for future use
UC0211	Luminal A	Stage I T1N0M0	<u>HLA-A*02:01</u> HLA-DR*04:01	16,000	Not done	Sequencing
UC0213	Luminal A	Stage II T2N0M0	HLA-A*24:02 HLA-DR*01:01	Not done	Not done	Tissue had too much lipid, no selection
UC0217	Basal-like	Stage I T1N0M0	HLA-A*29:02 HLA-DR*01:01	12,000	6,000	TIL RNA stored for future use
UC0221	Basal-like	Stage II T2N0M0	HLA-A*03:01 HLA-DR*07:01	100,000	94,000	TIL RNA stored for future use
UC0238	Luminal B	DCIS only	HLA-A*01:01 HLA-DR*03:01	Not done	Not done	Used to trouble shoot IFN- γ capture assay
UC0255	Unknown	Stage I T1N0M0	Pre-screen: HLA-A2+	Not known	Not known	Used to troubleshoot sequencing
UC0256	Basal-like	Stage I T1N0M0	<u>HLA-A*02:01</u> HLA-DR*13:04	256,000	76,000	Sequencing
UC0277	Basal-like	Stage I T1N0M0	<u>HLA-A2+</u>	Not done	Not done	Used to troubleshoot sorting specific populations for single cell PCR
UC0308	Luminal A	Stage II T1N1M0	<u>HLA-A2+</u>	8,000	4,000	Emulsion PCR, single cell sequencing
UC0323	Luminal B	Stage II T2N0M0	<u>HLA-A2+</u>	60,000	24,000	Emulsion PCR, single cell sequencing
UC0336	Luminal A	Stage I T1N0M0	<u>HLA-A2+</u>	24,000	12,000	Emulsion PCR, single cell sequencing

*Other patients were consented, but we did not obtain tissues

**RNA from CD4 TILs is being stored for future use

Patient	Subtype*	Stage*	HLA type	CD8 received	CD4 received	Sample use of CD8 TILs and LN**
C			<u>HLA-A*02:01</u> <u>HLA-DR*12:02</u>	T: 10,000 LN: 10,000	T: 10,000 LN: 10,000	Sequencing comparison of <i>ex vivo</i> and <i>in vitro</i> expanded TIL
D			<u>HLA-A*02:01</u> <u>HLA-DR*09:01</u>	T: 10,000 LN: 10,000	T: 10,000 LN: 10,000	Sequencing comparison of <i>ex vivo</i> and <i>in vitro</i> expanded TIL
E			<u>HLA-A*02:01</u> <u>HLA-DR*01:01</u>	T: 10,000 LN: 10,000	T: 10,000 LN: 10,000	Sequencing comparison of <i>ex vivo</i> and <i>in vitro</i> expanded TIL
BC41	Luminal A	Stage IIIA	<u>HLA-A2+</u>	T: 30,000 LN: 30,000	NA	Emulsion PCR, single cell sequencing
BC48			<u>HLA-A2+</u>	T: NA LN: 10,000	NA	Emulsion PCR, single cell sequencing
BC35	Luminal B	Stage IIA	<u>HLA-A2+</u>	T: 30,000 LN: NA	NA	Emulsion PCR, single cell sequencing
BC47			<u>HLA-A2+</u>	T: NA LN: 30,000	NA	Emulsion PCR, single cell sequencing
BC61	Luminal A	Stage IIA	<u>HLA-A2+</u>	T: 40,000 LN: 40,000	NA	Emulsion PCR, single cell sequencing
BC57	Luminal A	Stage IIB	<u>HLA-A2+</u>	T: 16,500 LN: 10,000	NA	Emulsion PCR, single cell sequencing
BC55	Her2+	Stage IV	<u>HLA-A2+</u>	T: 30,000 LN: NA	NA	Emulsion PCR, single cell sequencing
BC54	Luminal A	Stage IIIA	<u>HLA-A2+</u>	T: 10,000 LN: NA	NA	Emulsion PCR, single cell sequencing
BC67	Luminal A	Stage IIIA	<u>HLA-A2+</u>	T: 20,400 LN: 20,600	T: 23,000 LN: 37,000	Emulsion PCR, single cell sequencing
BC70	Basal	Stage IV	<u>HLA-A2+</u>	T: 50,000 LN: NA	T: 30,000 LN: NA	Emulsion PCR, single cell sequencing
BC71	Basal	Stage IA	<u>HLA-A2+</u>	T: 30,000 LN: 50,000	T: 30,000 LN: 100,000	Emulsion PCR, single cell sequencing
BC80			<u>HLA-A2+</u>	T: 5,000 LN: 25,000	T: 5,000 LN: 25,000	Emulsion PCR, single cell sequencing
BC81			<u>HLA-A2+</u>	T: NA LN: 25,000	T: NA LN: 25,000	Emulsion PCR, single cell sequencing
BC85			<u>HLA-A2+</u>	T: 10,000 LN: 10,000	T: 13,000 LN: 17,000	Emulsion PCR, single cell sequencing
BC75			<u>HLA-A2+</u>	T: 5,000 LN: 100,000	T: 5,000 LN: 100,000	Emulsion PCR, single cell sequencing
BC86			<u>HLA-A2+</u>	T: 47,500 LN: 50,000	T: 50,000 LN: 50,000	Emulsion PCR, single cell sequencing
BC87			<u>HLA-A2+</u>	T: 50,000 LN: 50,000	T: 50,000 LN: 50,000	Emulsion PCR, single cell sequencing
BC92			<u>HLA-A2+</u>	T-L: 10,000 T-R: 22,000 LN-L: 60,000 LN-R: 100,000	T-L: 7,000 T-R: 14,000 LN-L: 100,000 LN-R: 100,000	Emulsion PCR, single cell sequencing

*Subtype and stage for patients in Table 4

**RNA from CD4 TILs and LN is being stored for future use

Table 1. Breast cancer patient samples from UC AMC protocol and selected samples from the City of Hope protocol. HLA-A2+ samples are underlined.

Task 3e: Process patient samples (blood, TDLNs, tumor) and expand T cells ex vivo [to obtain the their TCR sequence].

Experiments to determine the efficacy of *ex vivo* T cell expansion were outlined in last year's Annual Report. Briefly, although expansion of T cells in culture provides us with more cells for analysis, the population dynamics changes such that same highly frequent clones present in the pre-expansion population are lost in the post-expansion population. Additionally, some V regions as well as VJ combinations dominate the post-expansion population that are negligible in the pre-expansion population. While between 29-63% of clones differ by <10 fold between the populations, there is no guarantee that these are tumor specific clones; even though some clones are highly frequent in the pre-expansion population and remain so following expansion. Furthermore, it is well established that tumor resident T cells are often dysfunctional, and when removed from the tumor microenvironment do not proliferate and instead die. Because of the observed skewing and the observation by numerous other groups concerning T cell growth following removal from the tumor, we have decided to pursue direct *ex vivo* sequencing. We started these studies sequencing the alpha and beta chains separately, which are summarized in the appendix and are waiting for one more figure before publication. Identification of the alpha and beta chains together will much better address our ultimate goals of identifying epitopes and mimotopes for bc immunotherapies.

Emulsion RT-PCR:

Presented here is (1) the emulsion PCR protocol, (2) experiments done to validate the technique, and (3) T cell receptor sequences from patient samples.

Emulsion RT-PCR protocol (adapted from Turchaninova et al¹):

Patient CD8+ or CD4+ T cells from PBMCs, lymph node, and/or tumor are pelleted and resuspended in 10 μ l of 150 mM NaCl. 40 μ l master mix containing 1x Qiagen One-Step RT-PCR buffer, 1x Qiagen Q-solution, 400 μ M dNTPs, 0.6 μ M C region primers, 0.6 μ M Stepout oligo, 0.22 μ M α and β V region oligos, 0.1 g/L BSA, 2 μ l Qiagen One-Step Enzyme mix, and 10 U RNaseOUT is added to this. To generate the emulsion, the Micellula Emulsion and Purification Kit is used. 300 μ l of emulsion phase is generated according to the manufacturer's protocol, added to the cells and master mix, and vortexed at high speed for 5 minutes at 4°C. The resulting emulsified reaction is subjected to reverse transcription (65°C 2 min, 50°C 30 min, 95°C 15 min) followed by 40 cycles of PCR amplification (94°C 30 s, 60°C 30 s, 72°C 2 min). The emulsions are broken and purified according to the Micellula protocol. The entire RT-PCR reaction is then amplified using nested C-region primers and non-overlapped TCR amplification is inhibited using blocking oligos. We use Long-Amp Taq, however any polymerase can be used for this reaction. A final nested C-region PCR is performed to add the adaptors and barcodes necessary for multiplex sequencing on the Illumina MiSeq.

Generated data are parsed into the individual multiplexed samples as fastq files and given to Kami Chiotti at OHSU for T cell receptor analysis using her program, Complete Clone, which is derived from the open source software MiTCR² and the IMGT database³. Paired sequence data are then returned to Denver for further analysis.

We first wanted to demonstrate that the protocol itself was working as expected. First, we examined the number of homologous pairs (i.e. α/α or β/β) (Figure 1). These sequences are deleted from the Complete Clone output because it only considers sequences with both alpha and beta chains. However, the frequency of mis-pairing is

important to understand the efficiency of the overlap PCR. The alpha frequency around 1% is low and acceptable since we are most interested clonotypes detected at higher frequencies. The frequency of beta mis-pairing, on the other hand, is high. Analyses of the data file showed that the majority of the sequences were from a single beta chain. These results suggest that this beta chain may be differentially expanded, an oligo may be mis-functioning in the overlap PCR, this sequence may “confuses” the analysis program, or some other kind of artifact. We continue to investigate this potential issue.

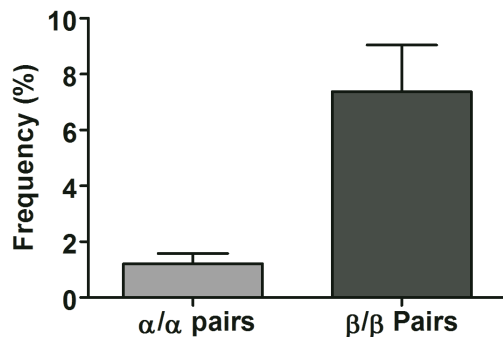


Figure 1. The frequency of sequences that were α/α or β/β pairs were quantified from 10 random samples from the 4 flow cells run to date. These sequences are never observed in the output analysis because Complete Clone only reports α/β TCR pairs.

Next we examined the frequency with which a given beta paired to a specific alpha when looking at all alpha pairs (**Figure 2**). If the reactions were not taking place in individual emulsion bubbles, then one would expect a given beta to be paired with all of the alphas resulting in a broad distribution. However, we see 70-95% of the top 20 betas pair with a single alpha over an average of 6 samples in 4 flow cells. These results suggest that the reactions are in fact taking place in individual emulsion bubbles. Turchaninova et al¹ estimated that the productive reaction bubbles are about 500 femtoliters in volume. Using the volume of the aqueous solution, we estimate that there are 40 times more of this size reaction bubble than cells added to the reaction. The results in **figure 2** suggest that we are not overwhelming the system by adding too many T cells and that the reaction bubbles are staying in tact during the first PCR.

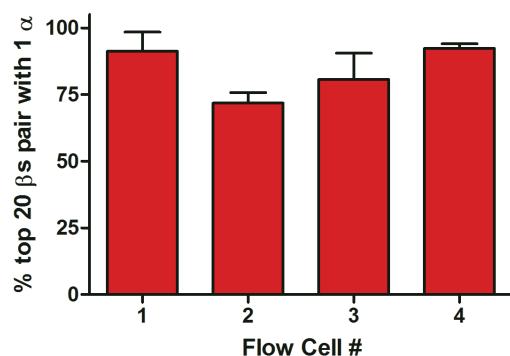


Figure 2 Most of the 20 most frequent beta CDR3 sequences paired with one alpha CDR3. 6 samples for each flow cell were analyzed.

Finally, we examined the recovery of a clonal T cell spiked into polyclonal PBMCs (**Table 2**). In this experiment, we dosed into PBMCs increasing numbers of 5KC cells (a mouse cell line that expresses a chimeric TCR: the V regions are from human and the C region is from mouse). We performed emulsion PCR on the samples, normalized to the amount of RNA per cell (because the hybridomas express more TCR RNA), and analyzed the sequences to determine the relative fraction of RNA molecules from the hybridoma verses the PBMCs. No emulsion control show the expected results.

% 5KC spike	H/H pairs	M/M pairs	H/M pairs	M/H pairs	Table 2. The fidelity and quantitative nature of the emulsion PCR were determined. H/H are human pairs, M/M are from the 5KC hybridoma, M/H and H/M are mis-paired TCRs. NE = no emulsion.
10%	89.30%	10.30%	0.32%	0.05%	
5%	94.00%	4.71%	0.21%	1.08%	
1%	98.20%	1.60%	0.22%	0.013%	
5% NE	95.12%	1.65%	0.71%	2.62%	

Next, we analyzed the TCRs of the patient samples acquired to date (summarized in Table 3). We determined if the same TCR sequences were present in T cells from tumor vs. peripheral blood of the same patient. To this end, we transformed the frequency data into rank (0-1) to scale every sample similarly despite the number of cells added to the reaction or the number of sequences obtained. Once we determined the rank values in the tumor and PBMCs, they were plotted and linked to each other (Figure 3, left). A weighted average (red line) was determined for each patient sample and plotted (Figure 3, right). For most samples (7/11) the weighted rank difference demonstrated a stark contrast of the TCRs derived from the tumor and PBMC of the same patient. This emphasizes that the tumor enriches these sequences and suggests that these TCRs recognize tumor-associated antigens. The next step will be to take the most frequent TCR pairs derived from the tumor and screen them for reactivity against the 5 known breast antigens and if necessary against the HLA-A2 peptide library in Task 6.

Table 3.

Tissue (CD8 T cells)	# samples sequenced	# cells/ sample ($\times 10^3$)	# reads/ sample ($\times 10^3$)	# $\alpha\beta$ pairs/ sample
PBMC	17	69 ± 14	342 ± 64	1349 ± 523
Lymph Node	9	34 ± 8	223 ± 149	364 ± 83
Tumor	13	23 ± 4	385 ± 94	926 ± 274

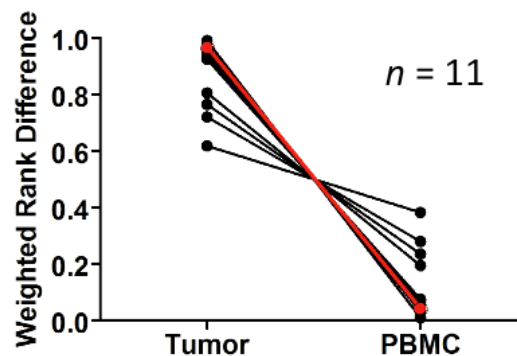
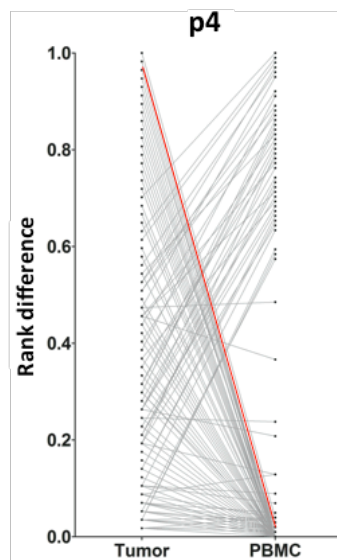


Figure 3: Differences in the tumor- and PBMC-derived TCRs. TCRs were ranked according to their frequency and the difference between the tumor and PBMC ranks plotted (left side, single patient example). On

the right, the weighted rank differences for 11 different tumor/PBMC pairs.

Because all of the patients are HLA-A*02:01+, we explored the possibility that there were common TCRs between the patients. We evaluated the tumor-derived TCR sequences and commonalities among them, shown in Table 4. A number of samples (see rows with more than 2 darker boxes) had shared TCRs that were highly frequent in their respective patient samples. Additionally, patients BC67 and UCD308 shared highly frequent TCRs, suggesting TCRs specific for similar antigens within these two tumor samples. Both of these samples, as well as BCs 41, 54, 57, and 61 are from patients with luminal A bc.

Share Count	BC41	BC35	BC61	BC57	BC55	BC54	BC67	BC70	BC71	UCD308	UCD323
11	0.300	0.444	0.056	0.088	0.238	0.056	0.949	0.053	0.158	0.991	0.057
9	0.250	0.800	0.346	0.018	0.143	0.070	0.747	0.789	-	0.897	-
9	0.100	0.200	0.009	0.053	0.024	0.028	0.911	-	0.053	0.949	-
9	0.050	0.178	0.804	0.018	0.286	0.070	0.873	-	0.053	0.906	-
8	1.000	0.133	0.009	0.140	0.024	0.028	0.861	-	-	0.111	-
8	0.900	0.600	0.112	0.368	0.095	-	1.000	-	0.053	1.000	-
7	-	0.111	0.019	0.088	0.143	-	0.886	-	-	0.966	0.029
7	0.300	0.222	0.056	1.000	0.500	-	0.013	-	-	0.299	-
7	0.150	0.067	0.009	0.053	0.048	-	0.785	-	-	0.923	-
6	0.650	0.578	-	0.649	0.262	-	0.443	0.947	-	-	-
6	0.200	0.022	-	0.035	0.071	-	0.962	-	-	0.983	-
6	-	0.311	0.047	0.035	-	-	0.013	0.105	-	0.026	-
6	-	0.089	0.019	0.053	0.095	-	0.899	-	-	0.880	-
6	-	0.067	0.019	-	0.048	0.028	0.937	-	-	0.889	-

Table 4. Sharing of TCRs across multiple bc patient-derived tumors. TCR sequences shared amongst multiple patients (indicated by the Count #) are shown per row (i.e. one row = one α/β TCR pair). The values are rank transformations of the frequencies and are shaded according to their rank value (dark higher, light lower). The scale is from 0-1 with 1 being the most frequent TCR. No value indicates that the TCR was not recovered from that patient's TCR sequences.

TCRs which are shared across multiple patients and also highly frequent present an attractive starting position for screening, thereby reducing the pool of potential TCRs to test by logically picking TCRs which are more likely to be restricted to HLA-A*02:01. As stated previously, we will use TCRs found here (of which there are only 14 displayed, a manageable number) to screen both the common BC antigens as well as the HLA-A*02:01 peptide library. It should be noted that TCRs found here, may or may not be inclusive of the TCRs we would wish to test from the data displayed in Table 4, as the data in Figure 5 does not discriminate against TCRs which are highly frequent in the blood. However, filtering out those sequences as a further step to narrow the potential TCRs to screen would be easy and appropriate.

Task 4. Identify and isolate anti-tumor T cells from TDLNs and tumor samples: months 1-40, Lee, Slansky, and Spellman

4b. Generate specific tumor antigen lysates from recombinant baculovirus-infected insect cells. *Currently expressing antigens in C1R cells since these cells can also be used as antigen presenting cells.

We plan to screen T cells against a panel of known BC antigens prior to screening libraries. If the T cells react with these known antigens, the road to antigen and mimotope discovery will be greatly shortened. Toward this goal, we have stably

transfected C1R:A2 cells (from Vic Engelhard at University of Virginia) with known breast cancer antigens HER2, NY-ESO-1, hTERT, CEACAM5, and MUC-1 (**Figure 4**). Evidence of tumor antigen expression is suggested by detection of Thy1.1 and breast cancer antigen-specific antibody staining. In addition to these well-studied breast cancer antigens, we are also considering adding some differentially expressed genes from the Spellman team.

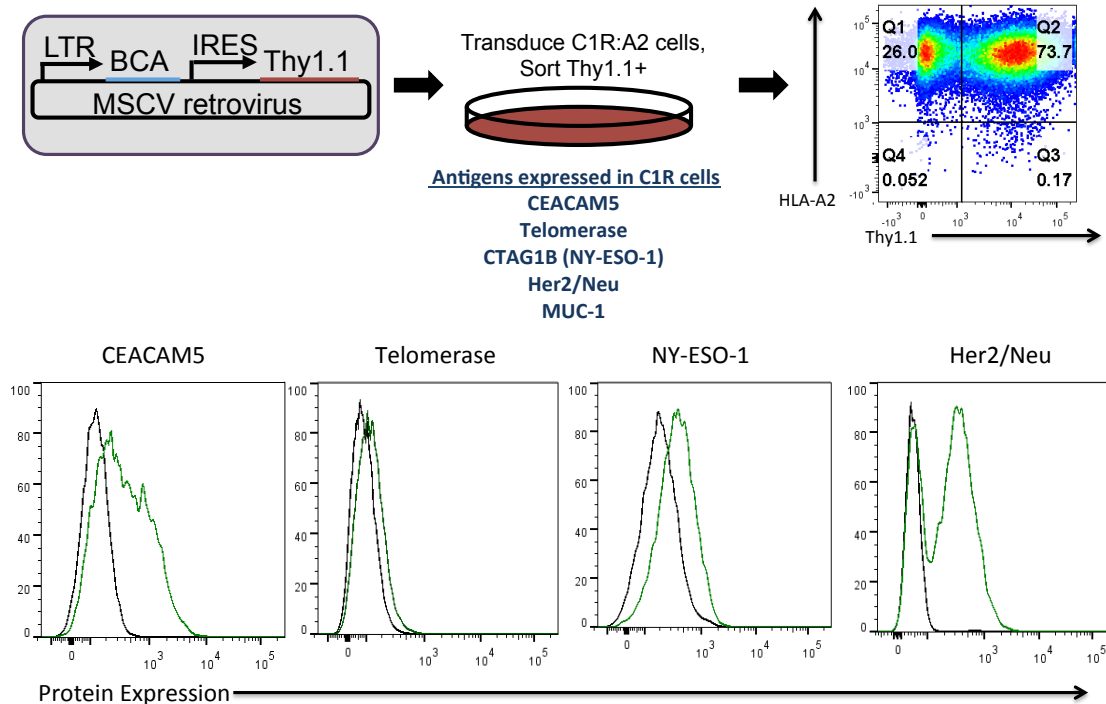


Figure 4. Generation of HLA-A2+ cell lines expressing specific bc antigens. Human C1R cells transfected with HLA-A2 are an EBV transformed B cell lymphoblast line and will process antigen for presentation of A2-specific epitopes. We detect both HLA-A2 and Thy1.1 in the cells when they are transduced with the various antigens. We also detect the bc-specific protein on the surface or inside the cell in each of the transduced cell lines (green histogram) compared to empty C1R cells (black histogram). The only cell line we do not have reliable protein staining for is MUC-1 because the flow antibodies to detect this protein mostly target the repeats of MUC-1, which were removed for transduction purposes. However, we detect reliable RNA signal for MUC-1 in the MUC-1 transduced cells and for all other genes (data not shown).

To confirm that the specific HLA-A*02:01-restricted tumor antigens are processed and presented by these cells, not just expressed, we are generating antigen-specific T cells. We are making these T cells by stimulating normal CD8 T cells from peripheral blood or normal spleen in culture with known peptides of breast cancer antigens loaded onto T2 cells in the presence of IL-2 and CD3 antibody. **Figure 5** shows how we are isolating the antigen-specific T cells. We will sub-clone the TCRs, insert them into hybridoma cell lines to make transfectomas, and use these to indicate antigen recognition on the C1R:A2-tumor antigen cell lines. This methodology will continue to be helpful when screening the peptide library as the total number of T cells may be limiting.

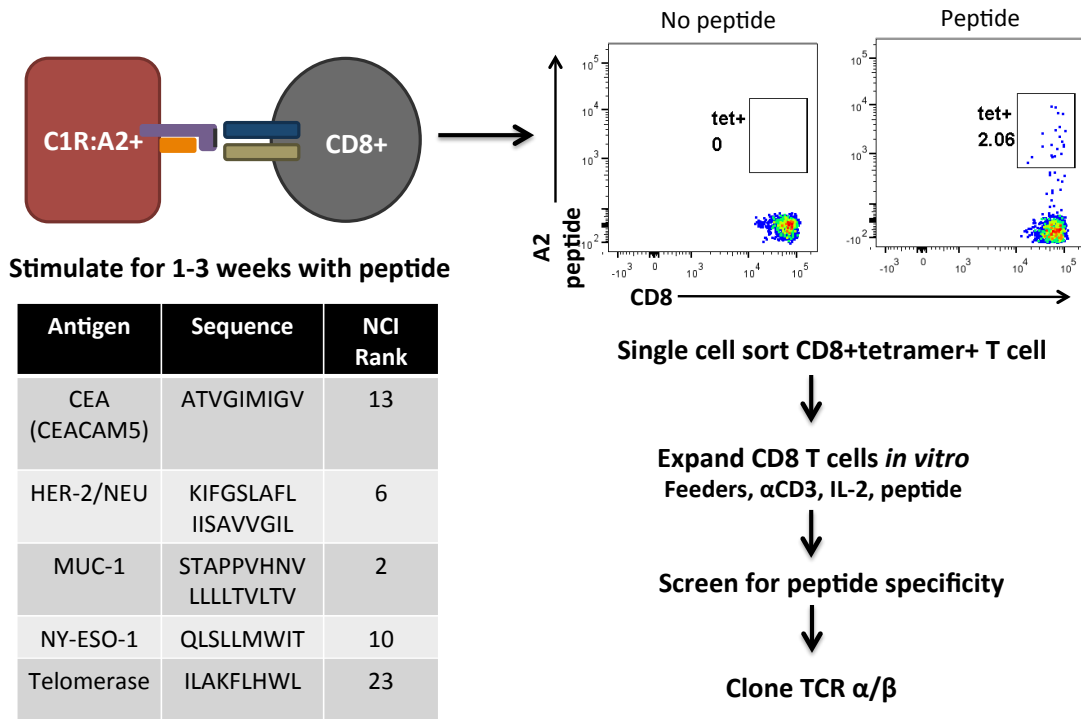
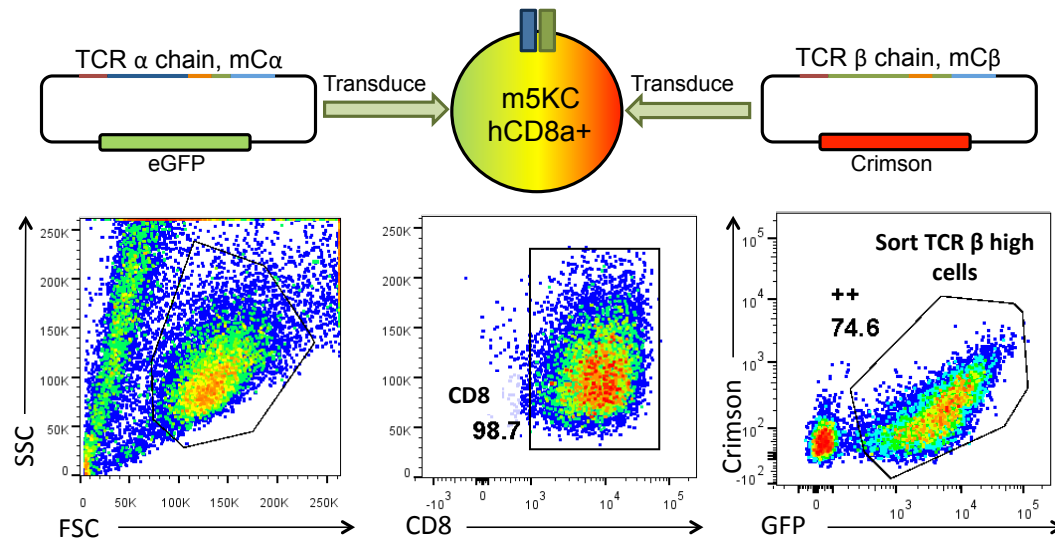


Figure 5. Generation of T cells specific to breast cancer antigens. CD8 T cells are positively selected from HLA-A2+ PBLs or normal spleen and stimulated with peptide-pulsed C1R:A2 cells at a 10:1 E:T ratio in the presence of IL-2 and αCD3. Cells are stimulated this way for 1-3 weeks and screened weekly for peptide specificity by tetramer staining. Tet+ cells are single cell sorted for clonal expansion. These cells are expanded so enough peptide-specific CD8 T cells are available to subclone the TCR α/β chains. The chart indicates the sequence of the most dominant peptides used for generation of the positive control clones and the ranking of the antigen by the NCI standards⁴.

The generation of transfectomas and an example of the process are detailed in **Figure 6**. Briefly, RNA is extracted from the T cells. A unique anchor is ligated to the 5' end of all transcripts and the RNA reverse transcribed into cDNA. PCR is performed with either an alpha or beta TCR reverse primer that binds to a conserved sequence in the constant region, and a forward primer specific to the linker. The reverse primer has a restriction site tail to aid in cloning. The PCR product is digested and inserted into plasmids used in transducing a T cell hybridoma that has little to no natural TCR expression, but expresses human CD8. The plasmids also contain GFP downstream of an IRES used for cell sorting in combination with the surface expression of the TCR molecule. TCR functionality can be assessed by binding to a potential target and measuring the release of IL-2 by ELISA.

In **Figure 6**, the most frequently identified TCR alpha and beta chains from patient UC0200 were synthesized and cloned into retroviral vectors. We paired the top α with the five top β sequences and the top β with the top 5 α sequences. Rank was determined by highest frequency of the sequence in the CD8 TIL of patient UC0200. Ten days after transfection the cells that expressed TCR beta chains were sorted and grown out. The next step with these transfectomas, along with the positive control breast tumor

antigen-specific T cell transfectomas, is to determine if they are activated by any of the C1R-A2 cell lines described in [Figure 4](#).



α sequence	TIL freq	Blood freq	LN freq
LVGDTLYSGTYKYI	4.98	0.005	0.86
ALSETDNQGGKL	1.33	0.007	0.73
GTEGGSNYKLT	1.25	0.001	0.23
VVANFGNEKLT	1.04	0	0.52
AVWPGGKLI	0.97	0.002	0.27
β sequence	TIL freq	Blood freq	LN freq
ASSLQNNQPQH	1.60	0.009	0.43
ATSEGIISTDTQY	1.56	0.020	1.05
ATSDGWRNQPQH	0.42	0.013	1.22
ASRTGSSYEQY	0.42	0	1.06
ASSVGVSNNQPQH	0.40	0.002	1.20

Figure 6. Creation of transfectomas using specific $\alpha\beta$ TCR pairs. TCR mRNA is isolated from T cells, linked to a known RNA sequence and reverse transcribed. PCR using primers specific to the constant region and to the known linker sequence creates a clonable TCR to put into a MSCV retroviral plasmid. The TCR α plasmid contains GFP and the TCR β contains Crimson, both under an IRES. The m5KC cells express human CD8. GFP+Crimson+CD8+TCR+ $\alpha\beta$ + cells can be sorted by flow cytometry. The $\alpha\beta$ pairings for UC0200 are listed with frequencies in CD8 TIL, PBL, and LN.

Task 6. Generate MHC/peptide baculovirus libraries: months 1-40, Slansky

6a. HLA-A*02:01

An HLA-A*02:01 peptide library has been constructed following the methods outlined in the previous Annual Report. The peptide library sequence constraints are X(VTPLIA)XXXXXX(LV), yielding a potential library size of 1.54×10^{10} possible peptide combinations. PCR amplicons were generated from the baculoviral DNA which encodes the library and sequenced on the HiSeq2500. [Figure 7](#) shows the distribution of amino acids at each position as well as the expected amino acids at each position following analysis of ~330 million sequence reads encoding 11 million peptides.

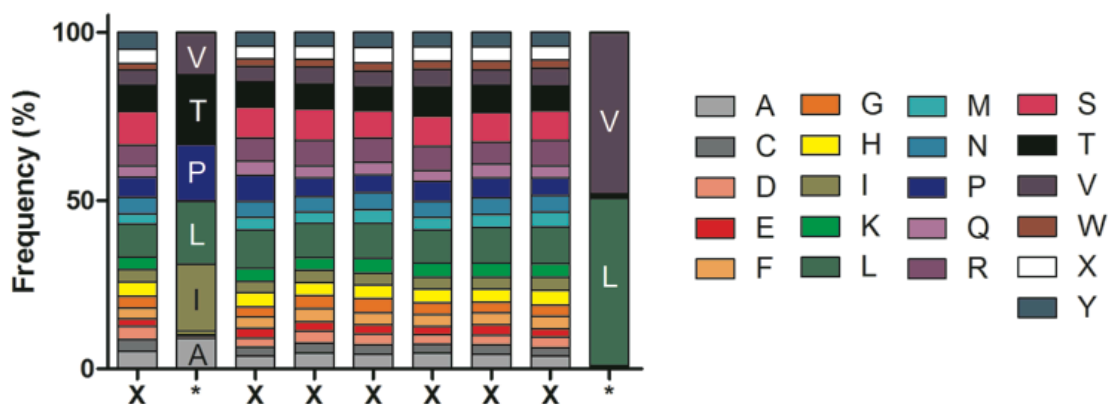


Figure 7. Sequencing of PCR amplicons of the peptide coding region of purified baculovirus DNA was performed to determine the amino acid distribution along the peptide. ~330 million DNA sequences were trimmed to the peptide sequence and translated. Unique peptide sequences were piled and the frequency of a given amino acid at position determined. Each amino acid is color-coded and although not visible every time, all 20 amino acids (as well as stop codons, X) are represented at the randomized positions. At the set positions, small numbers of contaminating amino acids were found due to mutation either from sequencing or from the viral polymerase during viral replication.

4. KEY RESEARCH ACCOMPLISHMENTS

- We obtained 19 HLA-A2 patient samples from the University of Colorado and 21 from the Lee Team at the City of Hope since the project started. We are acquiring sufficient samples to make progress.
- In addition to the positive control antigen-specific cell lines that we successfully generated in 2013, we now have a Muc1+ cell line to add to this list. We must still identify an antibody or TCR to show expression of Muc1.
- We developed a rapid high-throughput low-cost single-cell emulsion RT-PCR method for determination of alpha-beta TCR pairs.
- MiTCR and IMGT programs to identify the V, J, and CDR3 regions from the alpha and beta sequences separately. We worked with the Spellman team at OHSU to develop an algorithm that rejoins the sequences obtained from the same cell.
- We showed that the TCR sequences in the tumor are enriched versus those found in the blood.
- Using the emulsion PCR method, we fully analyzed the TCR sequences from CD8+ TILs from 11 HLA-A2+ bc patients.
- Analysis of these sequences showed that multiple TCRs are shared among patients.
- We generated a new HLA-A2-peptide library for screening. This library is randomized at all positions other than p2 and p9, the MHC-anchor residues. A

sample of the library was sequenced, of 330 million different sequences/11 million peptides were identified. Although this does not show full saturation of the library, all amino acids can be detected in the randomized positions.

5. CONCLUSION

In summary, in the last year, we have developed a rapid, high throughput, low-cost, single-cell emulsion RT-PCR method to identify dominant clonotypes that are found in the tumor but not to the same extent in matched PBMC samples (Figures 1-3, Tables 2-4). Soluble versions of these dominant TCRs will be used to screen (1) known antigens (Figure 4) and if these antigens are all negative, (2) a baculovirus-encoded peptide/MHC library. In the last year we have also generated a new highly diverse peptide library (Figure 7). The antigens that the TCRs identify can be added to the pool of “known antigen” to reduce the number of TCR clonotypes necessary to screen the library with. In the next two years (we predict that we will request a one year no-cost extension) the remaining part of this epitope/mimotope discovery procedure will be optimized to screen and boost the T cell responses detected in bc samples. These procedures may also contribute to identification other epitopes for other cancers.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS

PUBLICATIONS

Nothing to report, however, manuscripts describing (1) the TCR sequencing and antigen screening of patient 200 and (2) the emulsion PCR method are in progress.

The skewing paper discussed in the last annual report will not be submitted for publication, because the new method discussed within generates much more robust data.

PRESENTATIONS

Dec 4, 2013, Targeting effective T cells for cancer immunotherapy, Endocrine Research Conference, Anschutz Medical Campus (invited by Rebecca Schweppe).

Sept 12, 2014, A rapid high-throughput low-cost single-cell emulsion RT-PCR method for determination of alpha-beta TCR pairs, 15th Annual Colorado Immunology Conference, Breckenridge CO (invited by Jim Hagman).

Sept 20, 2014, Analysis of the T cell repertoire in breast cancer using emulsion single cell RT-PCR, Cancer Biology Graduate Program, 6th Annual Retreat, Snow Mountain Ranch CO (invited by Thomas Rogers, student).

Sept 29, 2014, Analysis of the T cell repertoire in breast cancer using emulsion single cell RT-PCR, Cardiff University School of Medicine, Wales (invited by Andrew Sewell).

Oct 8, 2014, Antigen-specific T cells responses to breast cancer. 9th International Conference of Anticancer Research, Sithonia, Greece (invited by John G. Delinassios).

Nov 8, 2014 (anticipated), Analysis of the T cell repertoire in breast cancer using emulsion single cell RT-PCR, Talk in Barriers to Successful Vaccines session at the annual meeting for the Society for Immunotherapy of Cancer (SITC) National Harbor, MD (invited by Jonathan Bramson).

Nov 11, 2014 (anticipated), Elimination of the bottlenecks in T cell receptor antigen discovery, Immunology Forum Seminar, Johns Hopkins University, Baltimore MD (invited by Jonathan Schneck).

7. INVENTIONS, PATENTS AND LICENSES

Nothing to report.

8. REPORTABLE OUTCOMES

- A. The most significant scientific advance and research tool that makes a meaningful contribution toward the understanding of how T cells of immune system interact with breast cancer was the development of the emulsion PCR method. With this method we can amplify the alpha and beta chains of the TCR together, which is necessary for the future experiment underway to identify the antigens that are recognized by these T cells.
- B. In October 2013 Jill Slansky visited the OHSU team to learn how they are mining databases to discover candidate tumor antigens and to discuss what is optimal from these sequences to generate potential shared antigens.

9. OTHER ACHIEVEMENTS

Nothing to report.

10. REFERENCES

1. Turchaninova MA, Britanova OV, Bolotin DA, Shugay M, Putintseva EV, Staroverov DB, Sharonov G, Shcherbo D, Zvyagin IV, Mamedov IZ, Linnemann C, Schumacher TN, Chudakov DM. Pairing of T-cell receptor chains via emulsion PCR. Eur J Immunol. 2013 43:2507-15.
2. Bolotin DA, Shugay M, Mamedov IZ, Putintseva EV, Turchaninova MA, Zvyagin IV, Britanova OV, Chudakov DM. MiTCR: software for T-cell receptor sequencing data analysis. Nat Methods. 2013. 10:813-4.
3. Lefranc M.-P. Unique database numbering system for immunogenetic analysis. 18: 1997. Immunology Today. 509.
4. Cheever MA, Allison, JP, Ferris, AS, Finn OJ, Hastings, BM, Hecht, TT, Mellman I, Prindiville SA, Steinman, Viner JL, Weiner LM, Matrisian LM. The prioritization of cancer antigens: A National Cancer Institute pilot project for the acceleration of translational research. Clin Cancer Res. 2009. 15:5323-5337.

11. APPENDICES

Introduction:

T cells are important in mediating and clearing infectious diseases, as well as in establishing life-long memory to the infectious agent. Although cancerous tissue is malignant and potential harmful to the host, the tissue comprising the tumor is derived from the host's own cells, making them inherently self. Within the thymus, mechanisms exist to suppress the generation of self-reactive T cells by deleting immature cells that react to strongly to self-peptides. As a result, tumors occupy a niche that is essentially hidden from the cellular immune response.

Although the tumor is self tissue, low affinity T cells specific to tumor antigens are not deleted in the thymus. However, because the affinity of these T cells is so low, binding of cognate antigen does not provide enough stimulation to cause the T cell to kill the target cell. It has been shown that low affinity T cells can be activated in the presence of peptide mimics, or mimotopes, of their cognate antigen. The activated cells are then primed to act against the cognate antigenic peptide-MHC and mimotope vaccination can protect animals from tumor challenge. In humans, the major hurdle is identifying the T cells that potentially target tumor antigens.

Recently, advances in high-throughput sequencing and multiplex PCR have made it possible to sequence large amounts of T cell RNA. Because T cells home to their cognate target, T cells circulating through the tumor and/or the draining lymph node are predicted to target antigens within the tumor. By comparing TCRs isolated from the tumor and/or lymph node to those derived from the peripheral blood of the same patient, potentially tumor reactive TCRs can be predicted based on their presence in the tumor and absence from the peripheral blood. Therefore, we hypothesized that by sequencing TCRs isolated from the tumor, blood, and lymph nodes of breast cancer patients, tumor reactive TCRs for mimotope development can be identified.

Results:

We collected draining lymph nodes (LNs), tumors, and peripheral blood mononuclear cells (PBMCs) from seven HLA-A*2:01+ breast patients as detailed in [Table A1](#).

Table A1:

Patient #*	Age	Subtype	Molecular Typing	Tissues Collected
p1	65	Luminal A	Stage 2b, ER+, PR+, Her2-	Tumor, Draining LN
p2	50	Luminal A	Stage 2b, ER+, PR+, Her2-	Tumor, Draining LN
p3	47	Luminal A	Stage 2a, ER+, PR+, Her2-	Tumor, Draining LN
p4	36	Luminal B	ER+, PR+, Her2+	Tumor, PBMC
p5	62	Luminal A	ER+, PR+, Her2-	Tumor, Draining LN, PBMC
p6	32	Luminal A	ER+, PR+, Her2-	Tumor, PBMC
p7	79	Basal-Like	ER-, PR-, Her2-	Tumor, PBMC

*Different patient samples than those shown above in this report.

CD8+ T cells were positively selected from each sample, the RNA was extracted and amplified, and submitted to iRepertoire for high-throughput TCR sequencing and analysis. The sequencing results are summarized in [Table A2](#).

Table
A2:

Patient #	Tumor				
	# Input	Alpha CDR3		Beta CDR3	
	Cells	Total	Unique	Total	Unique
p1	50,000	551,950	980	2,952,753	1,800
p2	38,000	2,085,776	2,344	2,463,014	1,444
p3	20,000	813,514	3,330	1,512,421	5,644
p4	4,000	708,105	548	5,013,033	1,922
p5	30,000	963,489	1,480	4,081,885	5,430
p6	16,000	963,211	616	1,515,860	1,957
p7	256,000	2,754,688	5,193	1,903,965	3,740

Patient #	Lymph Node				
	# Input	Alpha CDR3		Beta CDR3	
	Cells	Total	Unique	Total	Unique
p1	200,000	4,250,006	4,201	3,656,661	4,950
p2	1,240,000	1,564,348	4,371	467,900	4,102
p3	190,000	3,144,080	6,746	1,460,805	5,066
p4	-	-	-	-	-
p5	37,500	963,489	5,727	2,026,775	802
p6	-	-	-	-	-
p7	-	-	-	-	-

Patient #	Blood				
	# Input	Alpha CDR3		Beta CDR3	
	Cells	Total	Unique	Total	Unique
p1	-	-	-	-	-
p2	-	-	-	-	-
p3	-	-	-	-	-
p4	1,000,000	4,323,463	36,320	2,005,202	165,848
p5	1,000,000	4,503,650	22,521	3,118,472	13,798
p6	1,000,000	2,354,303	10,487	2,875,636	37,700
p7	1,000,000	4,751,007	38,391	1,627,181	24,748

“Total CDR3” refers to all CDR3 sequences including duplicate sequences whereas “Unique CDR3” refers to only unique CDR3 sequences. Any sequences which were present only once in the total CDR3 sequences were discarded because the predicted sequencing coverage suggests these are anomalies due to PCR and/or sequencing errors. In all cases but one, the number of total CDR3 sequences recovered surpassed the number of cells isolated, likely due to slight sampling error during pooling of the samples. Other reports have recovered similar numbers of unique CDR3

sequences per input cell suggesting we obtained adequate coverage to perform further analyses.

We first evaluated gross differences in CDR3 length between the tumor infiltrating T cells, the draining lymph node, and the peripheral blood (Figure A1). Overall, there were minimal changes to the CDR3 lengths when comparing the tissues to each other. The large error bar for the blood at 24 nt is due to the contribution of a single sample (p7) that had 40% of CDR3s of 24 nt length whereas the other samples were between 1.3 and 4.5%. This type of analysis is similar to immunoscope and spectratyping in that both technologies indicate gross changes in length. However, our analysis differs by using the sequence data as opposed to gel analysis and that out of frame sequences do not appear in our analysis since they are not reported as CDR3 sequences.

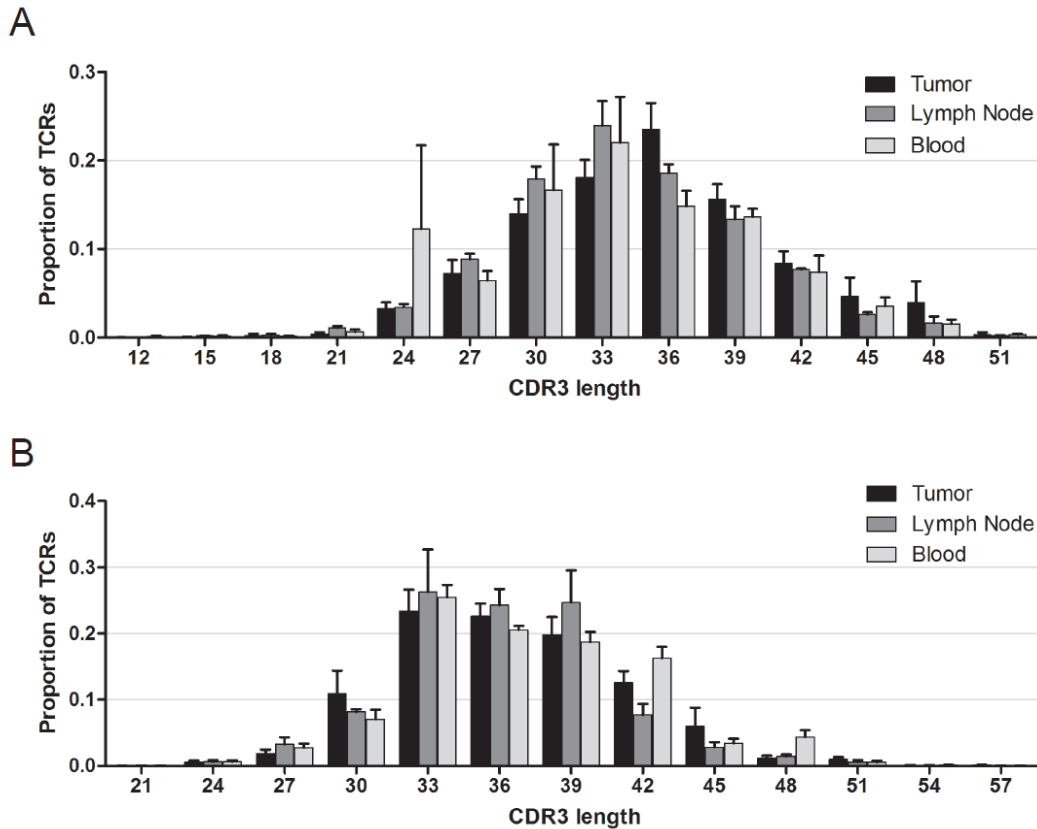


Figure A1: CDR3 lengths of TCRs derived from different T cell subsets from multiple patients. We also compared V and J segment usage between the populations. We plotted the proportion of specific V and J regions used in each group of samples either comparing tumor versus blood or tumor versus lymph node (Figure A2). Although some V and J regions had marked differences (indicated with the V or J region number), the majority of sequenced regions were similar between the compared populations.

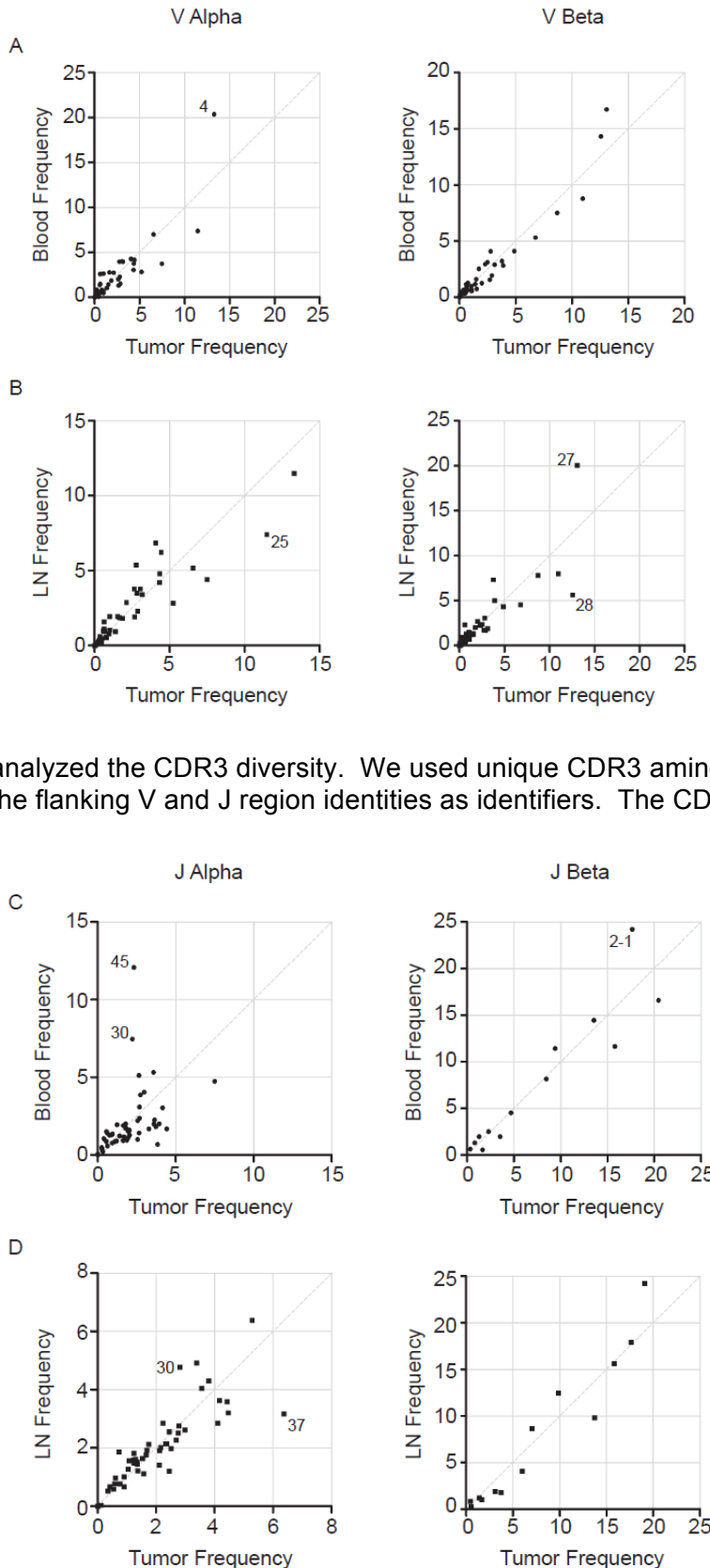


Figure A2: Frequency differences of the V (A and B) and J (C and D) regions when comparing the blood, tumor, and lymph nodes of breast cancer patients. V and J regions which had frequencies which differed by more than 5 are indicated. The angled line indicates where an equivalent value would occur.

Analysis of V regions, J regions, and CDR3 length suggested similarities in TCR gene usage the blood, lymph node, and tumor. However, differences emerged when we analyzed the CDR3 diversity. We used unique CDR3 amino acid sequences, coupled to the flanking V and J region identities as identifiers. The CDR3 sequences procured from tumor specimens were compared to those found in either the lymph node (Figure A3A) or the peripheral blood (Figure A3B). To visualize the differences, the frequency of individual CDR3 sequences from each tissue was converted to a rank and the two values were connected by a line. Thus, despite differences in maximal overall frequencies between populations, the positional information of a given CDR3 in the total population was preserved and the comparison populations were plotted on the same scale. We

calculated a weighted average of the rank differences to represent the overall trend change (Black lines and enumerations in **Figs A3A and B**). Because we focused on the differences between the TCRs of the tumor and non-tumor compartments, only the CDR3 sequences detected in the tumor were plotted. Although there was patient variability, the trend was that the CDR3 sequences from the tumor differed significantly from those in the lymph node and to a greater extent the blood.

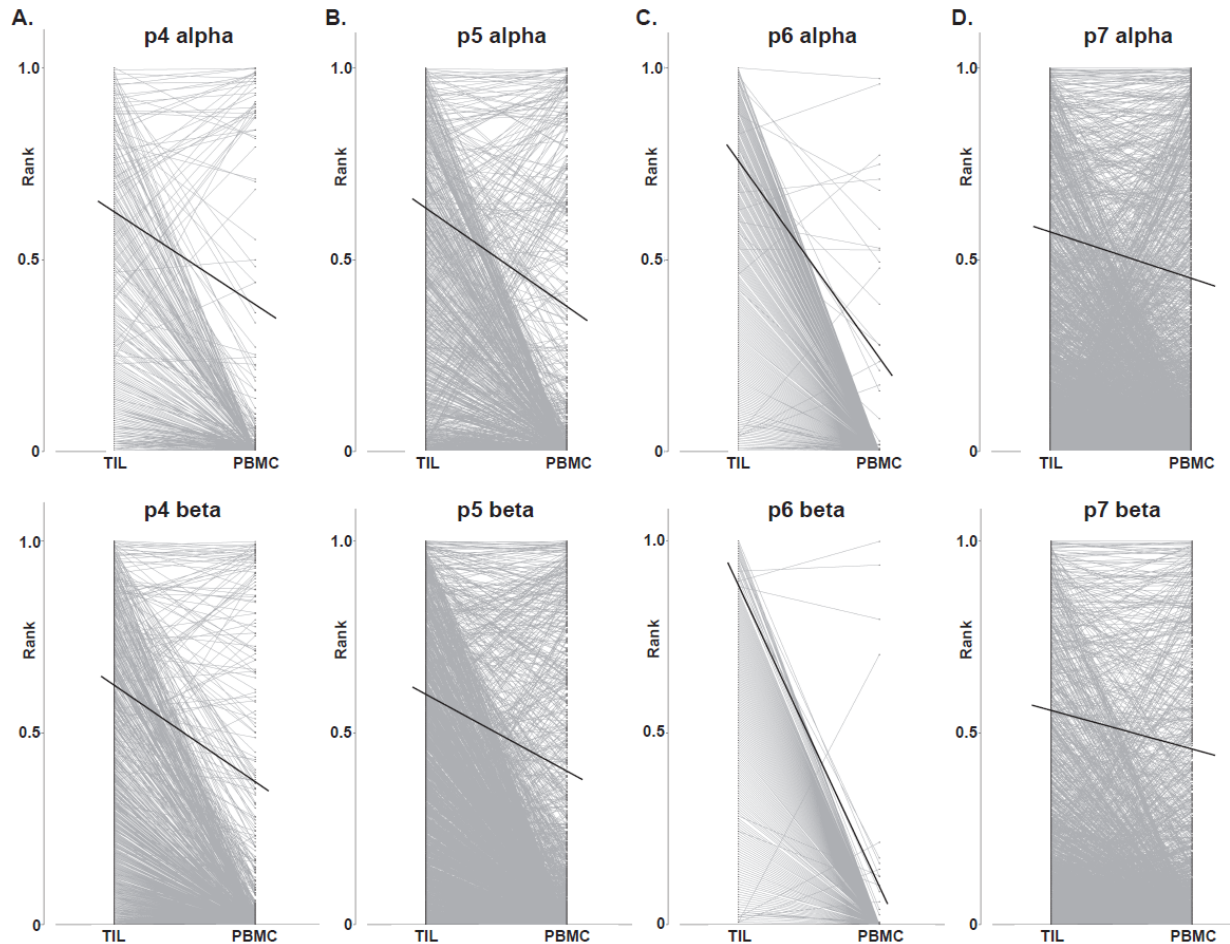


Figure A3: Rank differences between individual TCR sequences from either the alpha or beta chain derived from the tumor were plotted and compared to the rank of the same TCR in the PBMC from the same patient. The black lines indicate the overall weighted average of rank value difference.

We expected a number of common clones in the tumor and its draining lymph node. This trend was indeed reflected by the numerous nearly horizontal lines at the top of each graph (representing clones which were frequent in both populations). However, we also observed numerous clones which decreased in rank from the tumor to the lymph node, suggesting that although there was some conservation between populations, more clones decreased in frequency. This contrast was starker when we examined T cells from the tumor compared to the blood. While there were similarities, we more commonly observed increased divergence of the two populations as most readily observed in

patient #6. Patient #7 appears to have more similarity between the blood and tumor than the other patients had between the lymph node and tumor. This trend may be explained by increased vascularization of the tumor or as a difference in the gross pathology of the tumor as it is the only triple negative basal-like sample. Although many groups have isolated tumor-specific clones from the peripheral blood, our results indicate that these represent a small proportion of the cells present in the tumor. Similarly, although the draining lymph node is expected to have a large number of similar T cell clones to the tumor, we find that this is not necessarily the case, although not to the extent that differences exist between the blood and tumor. Thus, analysis of T cell clones present in the tumor and draining lymph node but not in the blood may be a good source of tumor specific T cells not normally identified by previous techniques.

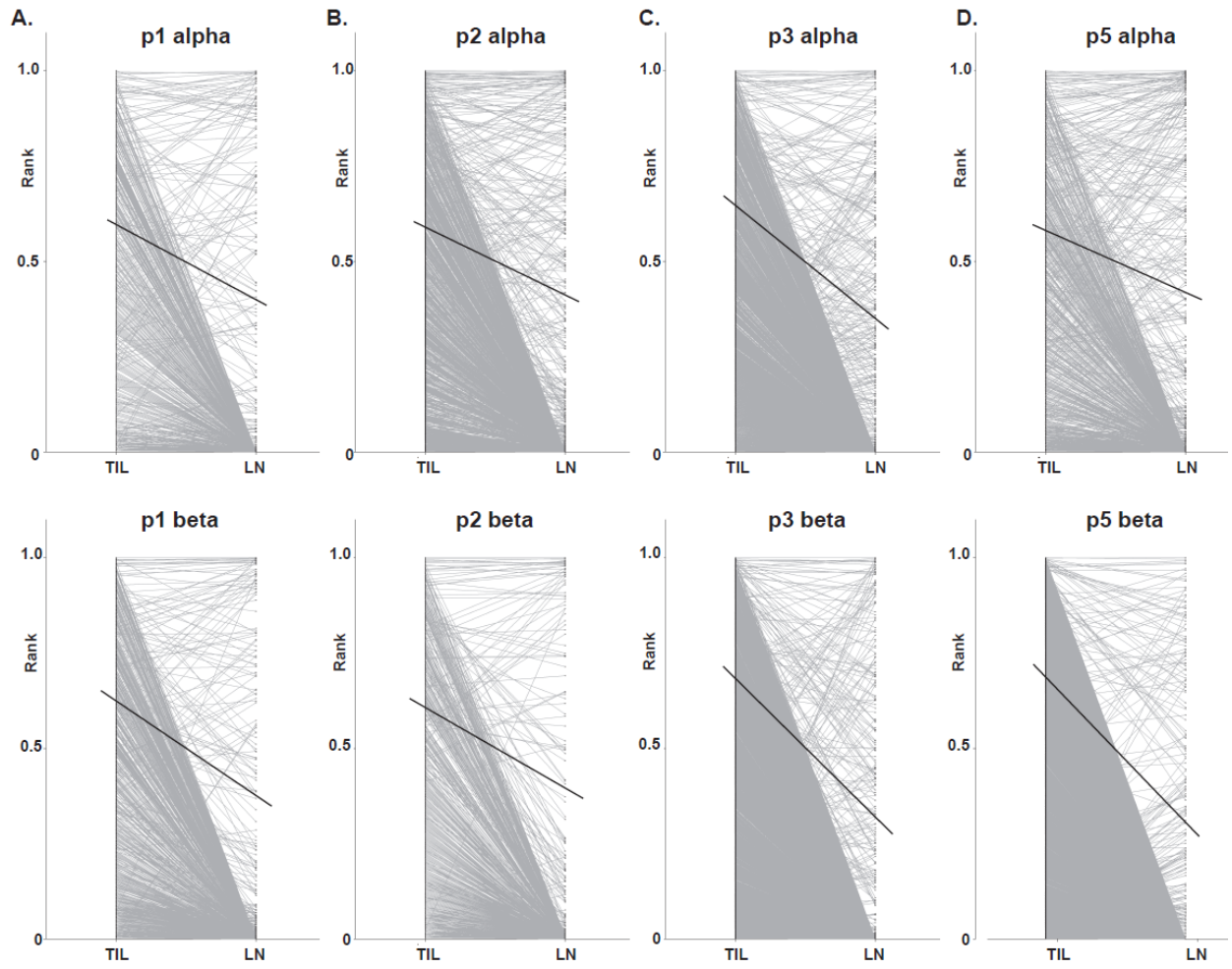


Figure A4: Rank differences between individual TCR sequences from either the alpha or beta chain derived from the tumor were plotted and compared to the rank of the same TCR in the LN from the same patient. The black lines indicate the overall weighted average of rank value difference.

We next asked if there were similarities in the TCR repertoire of the different tumors. We determined the number of similar CDR3 sequences between individual patients (Figure A5A) and the total number of shared CDR3 sequences among all patients (Figure A5B). The number of shared CDR3 sequences for both alpha (average

of 7.9, [Figure A5A](#)) and beta (average 11.7, [Figure A5A](#)) are shown. We identified a range of shared frequencies between patients, and patients shared at least one CDR3 sequence. When we looked at the overall number of CDR3 sequences that were shared amongst two or more patient tumors ([Figure A5B](#)), we observed a more limited repertoire of sequences common to 3 or 4 patients. Surprisingly, we detected a single alpha and beta CDR3 sequence common to all the tumor derived CDR3 sequences, possibly representing a public TCR which responds to breast tumors. Additionally, when we compared the lymph node ([Figure A5C](#)) and blood ([Figure A5D](#)) compartments, a large number of CDR3 sequences were found in two patients, with a larger overall number found in 3 patients in the blood than with the tumor samples ([Figure A5D](#)).

A

Alpha Beta	p1 (980)	p2 (2,344)	p3 (3,330)	p4 (548)	p5 (1,480)	p6 (616)	p7 (5,193)
p1 (1,800)		2	23	2	7	10	9
p2 (1,444)	7		5	2	5	4	16
p3 (5,644)	11	4		3	15	8	25
p4 (1,922)	25	4	4		5	1	7
p5 (5,430)	9	5	9	10		7	8
p6 (1,957)	64	5	9	16	16		3
p7 (3,740)	11	5	10	10	8	3	

B

Tumor Shared CDR3 sequences						
	Two	Three	Four	Five	Six	Seven
Alpha	93	9	5	-	-	1
Beta	140	12	4	2	-	1

C

LN Shared CDR3 sequences			
	Two	Three	Four
Alpha	156	7	1
Beta	33	4	-

D

Blood Shared CDR3 sequences			
	Two	Three	Four
Alpha	1411	57	3
Beta	996	63	4

In progress: The final experiments for this manuscript are currently ongoing. We have taken 5 TCR alphas and 5 TCR betas from p5 which were found to be highly frequent in the tumor and lymph node, and relatively infrequent (or absent altogether) in the blood derived TCR sequences. Those TCRs have been individually cloned and transduced into a murine T cell hybridoma (5KC), using the most frequent beta paired with all 5 alphas, and vice versa. These hybridomas will be screened for reactivity against the 5 common breast cancer antigens which were initially proposed (Fig 4, p 9). Additionally, soluble forms of the TCRs which no reactivity to the known antigens will be screened against the HLA-A*02:01 peptide library as described in Task 6.

Figure A5: Sharing of TCRs between patients. The number of TCRs shared among patient tumor derived TCRs is shown in A. The overall sharing of TCRs among multiple patients is shown in B-D for the tumor (B), Lymph node (C), and blood (D).